

# Effects of elevated atmospheric carbon dioxide on amino acid and $\text{NH}_4^+$ -N cycling in a temperate pine ecosystem

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## Abstract

Rising atmospheric carbon dioxide ( $\text{CO}_2$ ) is expected to increase forest productivity, resulting in greater carbon (C) storage in forest ecosystems. Because elevated atmospheric  $\text{CO}_2$  does not increase nitrogen (N) use efficiency in many forest tree species, additional N inputs will be required to sustain increased net primary productivity (NPP) under elevated atmospheric  $\text{CO}_2$ . We investigated the importance of free amino acids (AAs) as a source for forest N uptake at the Duke Forest Free Air  $\text{CO}_2$  Enrichment (FACE) site, comparing its importance with that of better-studied inorganic N sources. Potential proteolytic enzyme activity was monitored seasonally, and individual AA concentrations were measured in organic horizon extracts. Potential free AA production in soils ranged from 190 to 690  $\text{nmol N g}^{-1} \text{h}^{-1}$  and was greater than potential rates of soil  $\text{NH}_4^+$  production. Because of this high potential rate of organic N production, we determined (1) whether intact AA uptake occurs by *Pinus taeda* L., the dominant tree species at the FACE site, (2) if the rate of cycling of AAs is comparable with that of ammonium ( $\text{NH}_4^+$ ), and (3) if atmospheric  $\text{CO}_2$  concentration alters the aforementioned N cycling processes. A field experiment using universally labeled ammonium ( $^{15}\text{NH}_4^+$ ) and alanine ( $^{13}\text{C}_3\text{H}_7^{15}\text{NO}_2$ ) demonstrated that  $^{15}\text{N}$  is more readily taken up by plants and heterotrophic microorganisms as  $\text{NH}_4^+$ . Pine roots and microbes take up on average 2.4 and two times as much  $\text{NH}_4^+$   $^{15}\text{N}$  compared with alanine  $^{15}\text{N}$  1 week after tracer application. N cycling through soil pools was similar for alanine and  $\text{NH}_4^+$ , with the greatest  $^{15}\text{N}$  tracer recovery in soil organic matter, followed by microbial biomass, dissolved organic N, extractable  $\text{NH}_4^+$ , and fine roots. Stoichiometric analyses of  $^{13}\text{C}$  and  $^{15}\text{N}$  uptake demonstrated that both plants and soil microorganisms take up alanine directly, with a  $^{13}\text{C}:^{15}\text{N}$  ratio of 3.3:1 in fine roots and 1.5:1 in microbial biomass. Our results suggest that intact AA (alanine) uptake contributes substantially to plant N uptake in loblolly pine forests. However, we found no evidence supporting increased recovery of free AAs in fine roots under elevated  $\text{CO}_2$ , suggesting plants will need to acquire additional N via other mechanisms, such as increased root exploration or increased N use efficiency.

**Keywords:** alanine, amino acid, ammonium,  $^{13}\text{C}$  and  $^{15}\text{N}$ ,  $\text{CO}_2$ , FACE, microbial biomass N, organic nitrogen uptake

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## Introduction

In the absence of nutrient limitation, rising atmospheric carbon dioxide ( $\text{CO}_2$ ) is expected to increase carbon (C)

storage in terrestrial ecosystems, partially mitigating anthropogenic  $\text{CO}_2$  emissions. However, some analyses suggest that nutrient limitation is likely to constrain greater plant growth under elevated atmospheric  $\text{CO}_2$  (Hungate *et al.*, 2003). It is unlikely that the increased nitrogen (N) demand by plants under elevated atmospheric  $\text{CO}_2$  will be satisfied by inorganic N from microbial mineralization of soil organic matter (SOM) (Hungate *et al.*, 2003). Simple organic compounds, such

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as free amino acids (AAs), can provide an additional source of plant-available N (Schimel & Bennett, 2004) to support increased plant growth under elevated atmospheric CO<sub>2</sub>. It is unknown how AA availability will respond to elevated atmospheric CO<sub>2</sub>. Because simple organic compounds also supply bioavailable C, which fuels microbial activity, we expect that the cycling and demand of free AAs will differ from processes controlling inorganic N. Moreover, because N availability is important to C sequestration (Hu *et al.*, 2001; Gill *et al.*, 2002; King *et al.*, 2004), quantifying the importance of AAs in natural ecosystems and the response of AA cycling to elevated CO<sub>2</sub> is needed.

During the first 4 years of the Duke Forest Free Air CO<sub>2</sub> Enrichment (FACE) experiment, CO<sub>2</sub> fumigation stimulated net primary productivity (NPP) (Hamilton *et al.*, 2002). As a result, trees under elevated CO<sub>2</sub> required an additional 0.86 g N m<sup>-2</sup> yr<sup>-1</sup> compared with trees in ambient CO<sub>2</sub> plots (Finzi *et al.*, 2002). Net mineralization and belowground inorganic N cycling have not measurably changed, and therefore, do not contribute to the additional N requirement under elevated CO<sub>2</sub> (Finzi & Schlesinger, 2003). Nor has N<sub>2</sub> fixation increased under elevated CO<sub>2</sub> (Hofmockel & Schlesinger, 2007). The source of increased N supporting this enhanced NPP remains unclear, with organic N supply as an unexplored mechanism for meeting plant N demand. Trees of loblolly pine (*Pinus taeda* L.) support ectomycorrhizae, which have been proposed to produce proteolytic enzymes and take up organic N (Leake & Read, 1989). We hypothesize that under elevated CO<sub>2</sub>, increased production of roots and ectomycorrhizae may stimulate the availability and uptake of free AAs. We are not aware of any study that has investigated the uptake of organic N in warm temperate ecosystems, nor are we aware of any study that has investigated whether greater plant growth under elevated CO<sub>2</sub> will influence organic N uptake.

Our objectives were to determine: (1) if potential proteolytic enzyme activity and AA production is ecologically important in loblolly pine forests, (2) whether under field conditions loblolly pine assimilates intact AAs, a source previously unmeasured in this forest, (3) if cycling of AAs among soil pools is comparable with rates of NH<sub>4</sub><sup>+</sup> cycling, and (4) if elevated atmospheric CO<sub>2</sub> changes soil AA dynamics through interactions with C availability. To address our objectives, we added trace amounts of highly enriched <sup>15</sup>N and <sup>13</sup>C alanine (ala) and ammonium (<sup>15</sup>N only) to the soil *in situ* to compare the potential importance of intact AA and inorganic N uptake in forest plots exposed to ambient and elevated CO<sub>2</sub> concentrations. The organic horizon was the focus of our study because this layer contains the highest density of fine roots and fungal hyphae,

making it an important zone of N transformation and uptake. To understand the movement of organic N relative to inorganic N, we measured short-term cycling (hours to 1 month), retention, and distribution of labeled alanine (<sup>13</sup>C<sub>3</sub>H<sub>7</sub><sup>15</sup>NO<sub>2</sub>) and ammonium (<sup>15</sup>NH<sub>4</sub><sup>+</sup>) in plant, soil, and microbial pools.

## Materials and methods

### Study site

N cycling experiments were conducted at the Duke FACE site, North Carolina, USA. The site consists of six 30 m diameter plots in a loblolly pine (*P. taeda* L.) forest planted in 1983. Loblolly pine comprises 98% of the basal area of this forest (DeLucia *et al.*, 1999). At the time of this experiment, the canopy was ~20 m tall. Since August 1996, the experiment has maintained three control plots receiving ambient air, and three treatment plots receiving CO<sub>2</sub> enriched air (ambient CO<sub>2</sub> +200 μL L<sup>-1</sup>). For further details of the FACE experiment see Hendrey *et al.* (1999). Mean annual temperature is 15.5 °C, and mean annual precipitation is 1140 mm. The soils are deep and highly weathered clay loams, developed from igneous parent materials and are classified as Ultic Hapludalfs of the Enon series. They are moderately acidic with a pH of 5.75 (Finzi *et al.*, 2002). The soils are relatively infertile and NH<sub>4</sub><sup>+</sup> dominates inorganic N pools. Net nitrification rates are low, but highly variable and gross rates of nitrification have not been measured (A. C. Finzi, personal communication). In this forest, N is very tightly cycled and there is minimal loss of N through leaching (Lichter *et al.*, 2000). Our research focused on the O<sub>ea</sub> horizon, because it supports a dense mat of roots and fungal hyphae.

### Potential production rates and pools

Potential proteolytic and amidohydrolase enzyme activities were assayed seasonally on four samples from each plot. Organic horizon samples (O<sub>ea</sub>) were collected from 100 cm<sup>2</sup> areas, avoiding contamination from the mineral horizon. Samples were placed on ice and returned immediately to the lab for processing. Live roots were removed from each sample. A subsample of the root-free, organic horizon was oven-dried at 60 °C to determine gravimetric soil moisture content.

The remaining sample was used for enzyme analysis. The standard method for deamidizing enzymes is the amidohydrolase activity assay (Tabatabai, 1994). This method is used to determine NH<sub>4</sub><sup>+</sup>-N released by soil samples incubated with THAM buffer and toluene. Toluene increases the permeability of the microbial cell

membrane, allowing measurement of both intracellular and extracellular enzyme activity (Skujins, 1967). By substituting sodium acetate buffer for THAM buffer, we were able to use the proteolysis assay (Watanabe & Hayano, 1995) to concurrently measure potential gross production rates of AAs and  $\text{NH}_4^+$ . Six subsamples (3 g) of root-free  $\text{O}_{\text{ea}}$  horizon material were measured into six 40 mL centrifuge tubes, each with 10 mL of sodium acetate buffer. Next, 0.4 mL of toluene was added to inhibit microbial consumption of AAs and  $\text{NH}_4^+$ , and samples were shaken lengthwise during incubation periods of 1, 3, 5, 7, 24, and 48 h. At the end of each incubation period, 3 mL of trichloroacetic acid was added to arrest enzyme activity. Samples were centrifuged at 710 g for 5 min, and then filtered through a 0.5  $\mu\text{m}$  glass fiber filter.

Free AA concentration was determined by the *o*-phthalaldehyde and  $\beta$ -mercaptoethanol (OPAME) method (Jones *et al.*, 2002). The OPAME reagent does not react appreciably with sugars, organic acids, proteins, or humic acids, providing an accurate estimate of free AAs (Jones *et al.*, 2002). Aliquots of 0.1 mL of sample and standards were added to 3 mL of buffered reagent (100 mg *o*-phthalaldehyde, 10 mL methanol, 0.1 mL 3-mercaptopropionic acid, and 200 mL borate buffer, pH 9.5). A 200  $\mu\text{L}$  aliquot of each reaction mixture was pipetted into a Costar 96-well round-bottom, polystyrene, assay plate. AA concentration was determined by fluorescence with a 360 nm excitation filter and a 465 nm emission filter (35 nm bandwidth of each filter), using leucine as the AA standard.  $\text{NH}_4^+$  concentrations were analyzed colorimetrically (Lachat QuickChem FIA1 8000 Series, Lachat Instruments, Milwaukee, WI, USA). Production rates were calculated as the slope of the relationship between AA or  $\text{NH}_4^+$  production and time.

### AA profile

Before the field tracer experiment, 100  $\text{cm}^2$  organic horizon samples were collected from each FACE ring and brought immediately to the lab, where live roots were removed. Approximately 150 g of fresh organic horizon material was extracted in 450 mL of 0.01 M  $\text{CaCl}_2$  for 24 h at 25 °C. Samples were centrifuged at 1645 g for 10 min and filtered. Extracts were frozen and analyzed for individual AAs at the Duke University Medical Center using tandem mass spectrometry (Millington *et al.*, 1991).

### Tracer application

Our goal was to observe N cycling under field conditions using tracer techniques. Based on proteolysis results and on the abundance of individual AAs in

the field, low levels of highly enriched  $^{13}\text{C}_3\text{H}_7^{15}\text{NO}_2$  (*L*-alanine) and  $^{15}\text{NH}_4\text{Cl}$  were applied to small subplots within the Duke Forest FACE plots in April 2003. A 2.25  $\text{m}^2$  experimental plot was established in each FACE plot. The experimental plot in each FACE ring was equally divided by a polyvinyl chloride (PVC) sheet that extended into the mineral horizon.

Litter ( $\text{O}_i$ ) was carefully removed from the soil surface, and tracer was applied directly to the organic ( $\text{O}_{\text{ea}}$ ) horizon with a backpack sprayer (Perakis & Hedin, 2001). Isotope application consisted of a single dose of universally labeled  $^{13}\text{C}_3\text{H}_7^{15}\text{NO}_2$  (98%  $^{15}\text{N}$  and 98%  $^{13}\text{C}$ ) to one-half of each plot and  $^{15}\text{NH}_4\text{Cl}$  (98%  $^{15}\text{N}$ ) to the other half. The tracer was applied with enough water to simulate a 3.5 mm rain event typical for spring in North Carolina. Tracer N was added at a rate of 7  $\text{mg N m}^{-2}$  (0.125 mM solution), which is <5% of the extractable N pool (160  $\text{mg N m}^{-2}$ ).

### Sampling

Samples were taken from the  $\text{O}_{\text{ea}}$ , typically <5 cm deep. In each plot, 100  $\text{cm}^2$  samples of the  $\text{O}_{\text{ea}}$  horizon were removed before tracer application (natural abundance) and at 3 h, 1 day, 1 week, and 1 month after tracer application. Fresh samples were stored on ice and taken immediately to the lab for processing and analysis. Multiple 100  $\text{cm}^2$  samples were typically taken to obtain enough sample for chemical analysis. Each sample was independently analyzed for root biomass and bulk density, then composited for chemical analysis, detailed below. We traced N content and isotopic signature in the following pools: (1) exchangeable  $\text{NH}_4^+$ , (2) exchangeable dissolved organic N (DON), (3) chloroform ( $\text{CHCl}_3$ )-labile microbial N, (4) SOM N, (5) fine root N, and (6) soluble root N. For the alanine-tracer plots, pools 2–6 were also analyzed for C content and isotopic signature to measure intact AA recovery.

Live roots (<2 mm) were removed from each sample and soaked three times in 0.5 mM  $\text{CaCl}_2$  for 5 min to remove tracer absorbed to the root surface. Roots were then freeze-dried, weighed, and prepared for analysis of C, N,  $^{15}\text{N}$ , and  $^{13}\text{C}$  by elemental analysis-mass spectrometry (EAMS). Soluble C and N were extracted from dried fine roots in 10 mM phosphate buffer and also prepared for EAMS (Näsholm *et al.*, 1998; Bennett & Prescott, 2004; UC Davis Stable Isotope Facility, Davis, CA, USA).

Root-free organic soil samples were composited at the plot and N treatment level, and subdivided for elemental analysis and sequential extractions. Thirty grams of fresh soil were dried at 60 °C and measured for gravimetric soil moisture content. Dry samples were ground in a ball mill and weighed on the microbalance for

EAMS analysis (UC Davis Stable Isotope Facility). A second fresh subsample of 30 g was placed in a 120 mL bottle for sequential extractions.

#### Sequential extractions

A sequential extraction method was used to recover isotope tracer in SOM, microbial biomass, DON, NH<sub>4</sub><sup>+</sup> and fine roots (Holmes *et al.*, 2003). Thirty grams of root-free organic material were extracted in 0.5 M K<sub>2</sub>SO<sub>4</sub>, and the extract was passed through a 0.5 µm glass fiber filter. Ammonium and DON in the extract were determined by colorimetric analysis, then diffused for <sup>15</sup>N analysis according to (Stark & Hart, 1996). The glass fiber filter was removed from the filtration apparatus, returned to the sample bottle with the remaining moist soil, and analyzed for microbial biomass.

Microbial N was extracted via CHCl<sub>3</sub> fumigation extraction [adapted from Brookes *et al.* (1985)]. CHCl<sub>3</sub> was added directly to the soil pellet and filter remaining from the initial extraction. This adaptation has been shown not to differ significantly from vacuum fumigation (Witt *et al.*, 2000; Perakis & Hedin, 2001). Fumigated samples were extracted with 0.5 M K<sub>2</sub>SO<sub>4</sub>, and filtered through a 0.5 µm glass fiber filter. The microbial biomass extracts were digested by persulfate oxidation (Cabrera & Beare, 1993), analyzed for NO<sub>3</sub><sup>-</sup> colorimetrically, and diffused for <sup>15</sup>N analysis (Stark & Hart, 1996). Extracts were oven dried and analyzed for microbial biomass <sup>13</sup>C and <sup>15</sup>N (Colorado Plateau Stable Isotope Laboratory, Northern Arizona University). A recovery coefficient was not applied to correct for the fraction of microbial biomass that is not solubilized by the CHCl<sub>3</sub> extraction (Brookes *et al.*, 1985) due to the uncertainty associated with both temporal variations in the extractability of C and N from microbial biomass and the variability of incorporation efficiency into cytoplasmic vs. structural microbial components (Bremer & van Kessel, 1990). Therefore, our results represent a conservative estimate of the microbial biomass pool and isotope contents.

After completing NH<sub>4</sub><sup>+</sup>, DON, and microbial biomass extractions, the N within the remaining soil pellet was considered to represent soil organic N. The soil pellet was placed in an oven at 60 °C until dry, and then ground with a ball mill and prepared for EAMS (UC Davis Stable Isotope Facility). Diffusion samples were blank corrected (Stark & Hart, 1996), with percent recovery calculations used to follow tracer movement into soil and root components after tracer additions (Nadelhoffer *et al.*, 1999).

#### Calculations and statistical analysis

C and N content and <sup>15</sup>N and <sup>13</sup>C atom percentage in excess of the natural abundances (time 0) were used to

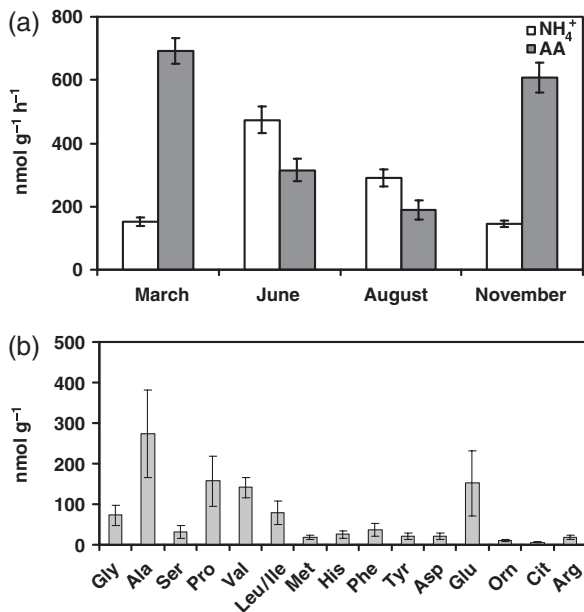
calculate µmol <sup>15</sup>N excess and µmol <sup>13</sup>C excess in samples from tracer addition plots. The percent of added label recovered in each pool was calculated by multiplying the atom percent excess of the pool by the pool size and dividing this value by the amount of label added in the field.

For statistical analysis the FACE plot was used as the experimental unit. For each date, all measurements on replicate samples within a plot were averaged for a single plot value (*n* = 6). Repeated-measures analysis of variance (ANOVA) was used to determine the effects of atmospheric CO<sub>2</sub> concentration (two levels: ambient, elevated), sample date (four levels: March, June, August, and November) and their interaction on NH<sub>4</sub><sup>+</sup> and free AA production rates (*n* = 12, six FACE rings × 2 N treatments). When production rates were significantly different among seasons, linear contrasts of the means, using student's *t post-hoc* tests, were used to compare production rates of free AAs and NH<sub>4</sub><sup>+</sup> during the four sampling dates (JMP Version 5.0.1a, SAS Institute, Cary, NC, USA).

Repeated measures ANOVA was also used to determine the influence of CO<sub>2</sub>, sample time (3 h, 1 day, and 1 week), N form (NH<sub>4</sub><sup>+</sup>, AA), and interactions on isotope excess in fine root and microbial biomass and percent recovery of <sup>15</sup>N in all soil fractions and <sup>13</sup>C in fine roots and microbial biomass. To measure uptake of alanine molecules by both fine roots and microbial biomass, linear regressions were used to evaluate the relationship between µmol excess <sup>13</sup>C and µmol excess <sup>15</sup>N, where a slope of 3 indicates the C : N of <sup>13</sup>C<sub>3</sub>H<sub>7</sub><sup>15</sup>N<sub>2</sub>, suggesting the molecule was taken up intact. A slope < 3 suggests extracellular mineralization prior to uptake or intact uptake and subsequent C respiration.

## Results

Proteolysis results indicate that mean annual potential AA production was significantly greater than potential NH<sub>4</sub><sup>+</sup> production (450 ± 40, 330 ± 30 nmol g<sup>-1</sup> h<sup>-1</sup>, *P* = 0.04), suggesting that free AAs were an important N pool throughout the year (Fig. 1a). Average potential NH<sub>4</sub><sup>+</sup> production was lower than AA production, ranging from 130 to 450 nmol N g<sup>-1</sup> h<sup>-1</sup> compared with 190–690 nmol N g<sup>-1</sup> h<sup>-1</sup>. Potential AA production rates were two and a half times higher than rates of NH<sub>4</sub><sup>+</sup> production during the relatively wet seasons of spring and fall (*P* < 0.001, Fig. 1a). During the summer, the production of NH<sub>4</sub><sup>+</sup> was 1.8 times greater than the production of AAs by proteolysis (*P* < 0.001, Fig. 1a). Time, N form (AA or NH<sub>4</sub><sup>+</sup>), and their interaction significantly affected N production (*n* = 12; *P* = 0.03, 0.009, and < 0.001). We detected no main or interaction CO<sub>2</sub> effects on AA or NH<sub>4</sub><sup>+</sup> mobilization. Free AAs in



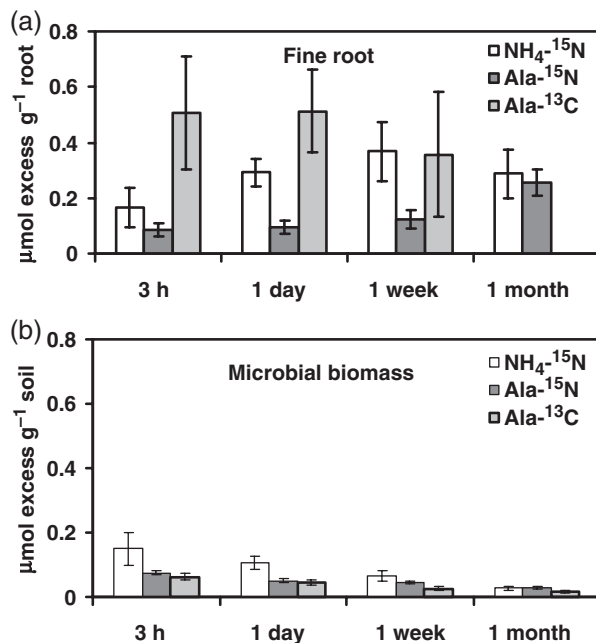
**Fig. 1** Mean ( $\pm$ SE) for (a) total ammonium (open bars) and total free amino acids (solid bars) produced during proteolysis experiments in the organic horizon at the Duke Forest free air CO<sub>2</sub> enrichment (FACE) site in 2003 and (b) free amino acid profile for FACE organic horizon soils before tracer addition (March 2003).

soil extracts were composed predominantly of nonpolar AAs, including alanine, proline, valine, leucine, and glycine (Fig. 1b). Neutral AAs were most abundant, with alanine representing 25% of the total pool.

Both forms of <sup>15</sup>N tracer were recovered in fine roots, but 2.7 times more ammonium than alanine was recovered during the first week of sampling ( $P = 0.04$ , Fig. 2a). After 1 month the <sup>15</sup>N recoveries of NH<sub>4</sub><sup>+</sup> and alanine were similar. Fine roots assimilated the <sup>13</sup>C during the first week (3 h, 1 day, 1 week), but after 1 month no <sup>13</sup>C was detected in fine root biomass (Fig. 2a). Because we have no <sup>13</sup>C recovery at 1 month, we used results from the 3 h, 1 day and 1 week to measure intact alanine uptake, CO<sub>2</sub> effects, and <sup>15</sup>N and <sup>13</sup>C distribution among soil pools.

To determine the extent of intact alanine uptake, we examined the relationship between <sup>13</sup>C and <sup>15</sup>N recovered in the soluble fraction of fine roots (Näsholm *et al.*, 1998). After 3 h the <sup>13</sup>C:<sup>15</sup>N ratio in fine roots was 3.3, similar to the ratio of these isotopes in the labeled alanine, suggesting the intact AA was taken up by roots ( $r^2 = 0.91$ ,  $P < 0.01$ ,  $y = 3.3048x - 0.028$ ).

Similar to fine roots, two times more NH<sub>4</sub>-<sup>15</sup>N than alanine-<sup>15</sup>N was recovered in microbial biomass ( $P = 0.03$ , Fig. 2b). Microbial biomass immobilized <sup>13</sup>C during all sample dates, suggesting intact uptake of alanine (Fig. 2b). Based on the <sup>13</sup>C:<sup>15</sup>N ratio of micro-

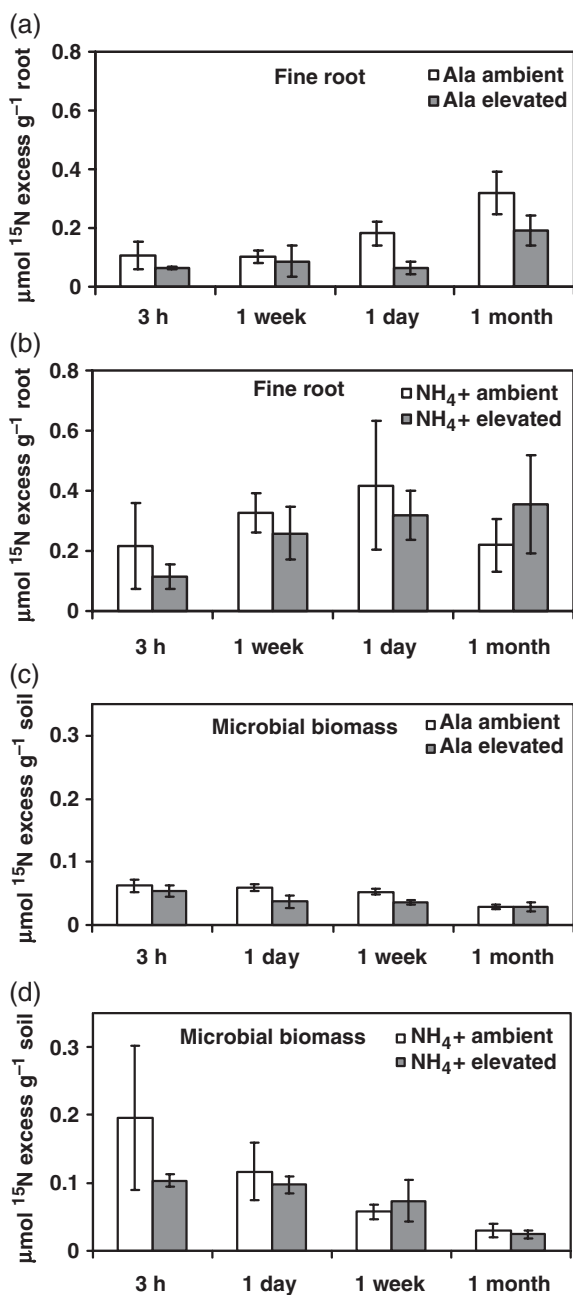


**Fig. 2** Isotope excess in (a) fine root biomass and (b) microbial biomass for <sup>15</sup>NH<sub>4</sub><sup>+</sup> (open bars), alanine <sup>15</sup>N (solid dark bars) and alanine <sup>13</sup>C (solid light bars). Bars represent mean ( $\pm$ SE),  $n = 3$ .

bial biomass after 3 h, it is likely that some intact uptake of alanine by microbes occurred, but the slope of this relationship was 1.5, which is significantly less than the C:N of alanine ( $r^2 = 0.68$ ,  $P = 0.04$ ,  $y = 1.4821x - 0.0237$ ).

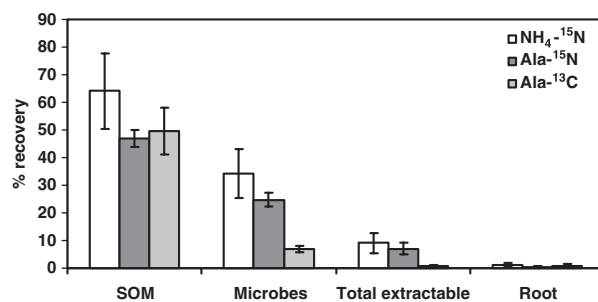
CO<sub>2</sub> exposure affected alanine recovery in roots, with significantly more alanine <sup>15</sup>N recovery under ambient CO<sub>2</sub> relative to elevated CO<sub>2</sub> ( $P = 0.03$ , Fig. 3a). CO<sub>2</sub> had no significant effect on NH<sub>4</sub><sup>+</sup> recovery in plants ( $P = 0.70$ , Fig. 3b). During the first week, more alanine <sup>15</sup>N under ambient CO<sub>2</sub> was recovered in microbial biomass than under elevated CO<sub>2</sub> ( $P = 0.03$ , Fig. 3c). No CO<sub>2</sub> effect was detected for microbial biomass NH<sub>4</sub><sup>+</sup> recovery (Fig. 3d). Similar to <sup>15</sup>N, more <sup>13</sup>C was recovered in microbial biomass under ambient CO<sub>2</sub> than elevated CO<sub>2</sub> ( $P = 0.08$ , data not shown). The recovery of stable isotopes in SOM, DON, and NH<sub>4</sub><sup>+</sup> fractions was unaffected by CO<sub>2</sub> treatment ( $P > 0.10$ , data not shown).

Both forms of N followed the same pattern of <sup>15</sup>N distribution among ecosystem pools. Total recovery (mean  $\pm$  SE) was  $105 \pm 14\%$  and  $82 \pm 14\%$  for NH<sub>4</sub><sup>+</sup> and alanine plots, respectively, and was not significantly different between N forms ( $P = 0.28$ ). Most <sup>15</sup>N was recovered in SOM followed by microbial biomass, DON, NH<sub>4</sub><sup>+</sup>, and roots (Fig. 4). Relative to alanine <sup>15</sup>N, more NH<sub>4</sub>-<sup>15</sup>N was recovered in fine roots ( $P < 0.01$ ), microbial biomass ( $P < 0.01$ ), and SOM ( $P = 0.01$ ) during



**Fig. 3** Fine root <sup>15</sup>N enrichment in (a) alanine tracer plots and (b) NH<sub>4</sub><sup>+</sup> tracer plots and microbial biomass <sup>15</sup>N enrichment of (c) alanine and (d) NH<sub>4</sub><sup>+</sup> tracer plots under ambient conditions (open bars) compared with elevated carbon dioxide (CO<sub>2</sub>) (solid bars).

the first week of the experiment (Table 1). We saw no significant differences between NH<sub>4</sub><sup>+</sup> and alanine <sup>15</sup>N in the pool of extractable DON or NH<sub>4</sub><sup>+</sup>. For both forms of <sup>15</sup>N, recovery was <2% for NH<sub>4</sub><sup>+</sup> and up to 15% for DON, indicating tight cycling and low availability of inorganic and organic N in this ecosystem. We found no significant CO<sub>2</sub> effect on percent recovery of <sup>15</sup>N or <sup>13</sup>C tracer for any soil fraction.



**Fig. 4** Average recovery of <sup>15</sup>NH<sub>4</sub><sup>+</sup> (open bars), Alanine <sup>15</sup>N (solid dark bars) and Alanine <sup>13</sup>C (solid light bars) 3 h after tracer addition. Bars represent mean (±SE), *n* = 6. For both forms of tracer, recovery was greatest in pools in the following order: SOM > microbial biomass > DON > fine roots. SOM, soil organic matter; DON, dissolved organic nitrogen.

## Discussion

Elevated atmospheric CO<sub>2</sub> experiments demonstrate that increased NPP may enhance C storage in forest ecosystems. In the absence of increased N-use efficiency by plants, additional N will be required to sustain C sequestration (Finzi *et al.*, 2002; Gill *et al.*, 2006). Elevated atmospheric CO<sub>2</sub> has consistently augmented NPP in the Duke FACE forest, requiring N uptake in excess of the measured inorganic N supply (Finzi *et al.*, 2002). Increased root and ectomycorrhizal growth under elevated CO<sub>2</sub> could enhance AA consumption, providing an additional source of bioavailable N to support increased plant growth and ecosystem C storage under elevated CO<sub>2</sub>. By measuring inorganic and organic N production and cycling, we investigated the importance of AAs relative to NH<sub>4</sub><sup>+</sup> under current and future atmospheric CO<sub>2</sub> conditions. Our results demonstrate that annual rates of potential AA production were greater than potential NH<sub>4</sub><sup>+</sup> production, and both loblolly pine and heterotrophic microorganisms can assimilate alanine intact. However, AA uptake by overstorey trees is not stimulated by elevated atmospheric CO<sub>2</sub>, indicating an alternative mechanism will be required to support enhanced plant N demand.

Over the course of our experiment, inorganic N was more accessible than organic N for loblolly pine roots as demonstrated by significantly greater recovery of NH<sub>4</sub><sup>+</sup>-<sup>15</sup>N than of alanine-<sup>15</sup>N in fine roots (*P* < 0.01, Fig. 2a). Despite higher potential AA production rates, 2.4 times more NH<sub>4</sub><sup>+</sup>-<sup>15</sup>N than alanine-<sup>15</sup>N was recovered in fine roots on average during the first week of our experiment. In a study of arctic plants, field uptake of inorganic <sup>15</sup>N was at least twice that of AA-<sup>15</sup>N (Nordin *et al.*, 2004). Other field studies have shown similar root uptake of glycine and NH<sub>4</sub><sup>+</sup> in boreal forests (Näsholm *et al.*, 1998; McFarland *et al.*, 2002) or wheat plants (Näsholm *et al.*, 2001).

**Table 1** Means ( $\pm$ SE) percent recovery of  $^{15}\text{NH}_4^+$ -N, Alanine- $^{15}\text{N}$  and Alanine- $^{13}\text{C}$  in soil organic matter (SOM), microbial biomass (MB), total extractable N and fine roots over the time course of the experiment

	SOM	MB	Extractable N	Root
$^{15}\text{N NH}_4^+$				
3 h	64.05 (13.73)	34.23 (8.90)	9.09 (3.56)	1.26 (0.59)
1 day	78.66 (17.17)	38.51 (9.05)	10.63 (3.42)	1.84 (0.37)
1 week	54.93 (20.11)	25.42 (7.82)	8.26 (2.92)	2.38 (0.77)
1 month	39.21 (3.82)	8.32 (0.43)	3.63 (0.56)	1.95 (0.60)
$^{15}\text{N Alanine}$				
3 h	46.90 (3.19)	22.47 (2.24)	7.02 (2.12)	0.57 (0.19)
1 day	38.09 (6.04)	14.96 (2.18)	5.66 (1.28)	0.81 (0.37)
1 week	39.77 (6.03)	13.60 (1.55)	5.38 (1.06)	0.87 (0.28)
1 month	37.64 (5.25)	8.84 (1.14)	3.82 (0.40)	1.82 (0.56)
$^{13}\text{C Alanine}$				
3 h	49.63 (8.32)	6.94 (1.17)	0.70 (0.28)	0.96 (0.42)
1 day	83.77 (28.78)	5.33 (0.96)	0.85 (0.23)	1.21 (0.45)
1 week	57.56 (18.55)	2.93 (0.67)	nd	0.66 (0.42)
1 month	8.26 (12.77)	1.88 (0.42)	nd	0.00 (0.67)

nd indicates no data.

AAs may be an important source of N for loblolly pines. Although isotope recovery in fine roots is not a true measure of plant N uptake, results from our field experiment suggest, that given equal pools, alanine recovery is at least 40% of  $\text{NH}_4^+$  recovery. As our proteolysis data demonstrate, pools of free AAs and  $\text{NH}_4^+$  are seldom equal. This will likely affect the relative importance of AAs in fulfilling plant N demand. During seasons when potential AA production exceeds potential  $\text{NH}_4^+$  production, such as in the spring and fall (Fig. 1a), AAs likely provide a more important source of labile N. It is possible that colorimetric analysis underestimates  $\text{NH}_4^+$  concentration due to interference by organic compounds (Schjoerring *et al.*, 2002) in which case potential rates of  $\text{NH}_4^+$  production may exceed AA production throughout the year. Translating field tracer results to N budget calculations is not straight forward, because our experimental design did not include calculations of  $^{15}\text{N}$  dilution from endogenous soil N. Greater potential AA production rates may result in dilution of the stable isotope signatures (Jones *et al.*, 2005). Consequently, our results may underestimate alanine uptake by plant roots. Even with a conservative estimate, our results demonstrate that AAs provide an important source of bioavailable N for loblolly pine.

Intact recovery of alanine in fine roots suggests N acquisition can occur independent of N mineralization. Fine root recovery of intact alanine over the short-term (<1 month) was verified by the positive relationship of excess  $^{13}\text{C}:^{15}\text{N}$  in the soluble fine root fraction. The slope of this relationship was approximately 3, indicating that, on average, three  $^{13}\text{C}$  were assimilated for each  $^{15}\text{N}$  (i.e. the  $^{13}\text{C}:^{15}\text{N}$  ratio in fine roots matches the

molecular composition of alanine tracer ( $^{13}\text{C}_3\text{H}_7^{15}\text{NO}_2$ ), which has a  $^{13}\text{C}:^{15}\text{N}$  ratio of 3:1). In fact, this relationship suggests that little  $^{13}\text{C}$  was respired, and all alanine recovered in fine roots was indeed the intact dual labeled AA. Although some studies have reported considerable root respiration of simple AAs during tracer experiments in boreal forests (Näsholm *et al.*, 1998; Näsholm & Persson, 2001; Bardgett *et al.*, 2003; Henry & Jefferies, 2003), other studies have shown substantial intact uptake, similar to our results (Näsholm *et al.*, 1998; McFarland *et al.*, 2002). Taken together, these results clearly indicate that AA uptake is an important part of the N cycle in a variety of ecosystems, including southern pine forests.

Similar to fine roots, soil microorganisms were also able to take up alanine. There was a significant positive relationship between  $^{13}\text{C}$  and  $^{15}\text{N}$  recovery in microbial biomass N. The slope was 1.5, suggesting that either a portion of the  $^{13}\text{C}$  was respired, or some of the alanine was mineralized extracellularly and recovered as  $^{15}\text{N}$ -ammonium (Näsholm & Persson, 2001), resulting in a  $^{13}\text{C}:^{15}\text{N} < 3$ . Endoenzymes released from disintegrating cells may persist, resulting in extracellular mineralization (Tabatabai, 1994). If extracellular mineralization occurred, the available  $^{15}\text{NH}_4^+$  would likely affect the fine root  $^{13}\text{C}:^{15}\text{N}$  ratio. On average  $0.5 \mu\text{mol excess } ^{13}\text{C g}^{-1}$  fine roots was recovered during the first week (Fig. 2a), indicating microbial respiration rather than extracellular mineralization. However, without direct measurements of respiration, we are unable to confirm  $^{13}\text{C}$  respiration rates.

Although alanine was an important N source to roots and microorganisms, we did not detect a  $\text{CO}_2$  stimula-

tion effect in alanine recovery. Significant increases in live fine root mass under elevated CO<sub>2</sub> have been detected in the Duke Forest soils (Matamala & Schlesinger, 2000), suggesting a greater biomass of ectomycorrhizal symbionts to exploit limiting resources from soil. However, we did not find greater potential proteolytic enzyme activity or greater AA recovery under elevated atmospheric CO<sub>2</sub>. Results from our tracer experiment suggest that elevated atmospheric CO<sub>2</sub> does not enhance AA cycling through plants and microorganisms in this ecosystem. Both pine tree roots and soil microorganisms assimilated significantly more alanine-<sup>15</sup>N in ambient CO<sub>2</sub> than in elevated CO<sub>2</sub> 1 week after tracer application (Fig. 3a and c), which is surprising, considering there is greater ecosystem N demand under elevated CO<sub>2</sub> (Finzi *et al.*, 2006). We assumed no differences in transport of the N forms from hyphae to roots or from roots to xylem. Laboratory studies of mycorrhizal-free Scots pine (*Pinus sylvestris* L.) indicate that alanine-<sup>15</sup>N uptake and transport to needles is equal to that of <sup>15</sup>NH<sub>4</sub><sup>+</sup> (Persson *et al.*, 2006). Because we did not measure transport explicitly, we cannot accurately calculate total uptake rates. Instead our data provide an approximation of the potential importance of each form of N to meet loblolly pine N demand (Finzi *et al.*, 2002).

Although more alanine was recovered (μmol <sup>15</sup>N excess g root<sup>-1</sup>) in fine roots under ambient CO<sub>2</sub> relative to elevated CO<sub>2</sub>, there is significantly more root biomass under elevated CO<sub>2</sub>. Consequently, trees may be acquiring equal quantities of alanine under both CO<sub>2</sub> treatments. When root uptake is analyzed at the ecosystem scale, considering the pool of fine roots (% recovery <sup>15</sup>N), we find no significant differences in alanine-<sup>15</sup>N assimilation due to the elevated CO<sub>2</sub> treatment. Our results indicate that root-specific responses are sensitive to C availability, however elevated CO<sub>2</sub> is not likely to affect AA (e.g. alanine) uptake at the ecosystem scale.

The largest biotic sink for available N throughout our experiment was microbial biomass, indicating microbial uptake was an important mechanism for retaining bioavailable N in this forest ecosystem. Three hours after application, 34 ± 8.9% (mean ± SE) of the <sup>15</sup>NH<sub>4</sub><sup>+</sup> and 24 ± 2.5% of the alanine-<sup>15</sup>N was recovered in microbial biomass N. These results are similar to data from an Alaskan floodplain forest, in which <sup>15</sup>N recovery in microbial biomass was 46% and 31% under NH<sub>4</sub><sup>+</sup> and glycine treatments, respectively (McFarland *et al.*, 2002). Other studies have demonstrated that microbial biomass is a stronger sink than roots in <sup>15</sup>NH<sub>4</sub> field experiments (Zak *et al.*, 1990; Schimel & Chapin, 1996; Perakis & Hedin, 2001) and pot studies (Henry & Jefferies, 2003). However, interpreting microbial biomass measurements in ectomycorrhizal systems is com-

plicated, because fungal hyphae comprise a large portion of the microbial biomass N, but functionally may represent a fraction of root N. Symbiotic fungi may initiate nutrient transport to the root system, with N initially retained in the hyphal network and passed on to the plant over longer time periods (Näsholm & Persson, 2001). The decreasing <sup>15</sup>N microbial signal (Fig. 2b) and increasing fine root <sup>15</sup>N signal (Fig. 2a) during the course of our experiment may be due to transport of N from hyphae to fine roots or from turnover of microbial biomass <sup>15</sup>N. Both would contribute to the low <sup>13</sup>C: <sup>15</sup>N signal in fine roots of alanine tracer treatment after 1 month.

Although AAs are an important source of N to loblolly pine, we found no evidence to suggest that elevated atmospheric CO<sub>2</sub> increases the uptake of AAs by the fine roots of these trees. Therefore, AA uptake is not likely to supply plants with the additional N required under elevated CO<sub>2</sub>. Our research clearly demonstrates that AAs are a substantial pool of plant available N in this temperate pine forests. Annual potential AA production was 1.4 times greater than NH<sub>4</sub><sup>+</sup> production, providing an important source of labile soil N. Our data suggest that fine roots of loblolly pine, as well as heterotrophic microorganisms can take up alanine intact. In the absence of pool dilution calculations and transport rates of various N forms, it is not possible to calculate specific uptake rates of NH<sub>4</sub><sup>+</sup> and alanine in plants and microorganisms. Nonetheless our data indicate that alanine is potentially an important source of bioavailable N, contributing to the N economy of temperate pine forests. Our study contributes to the growing body of evidence suggesting that free AAs are a significant component of terrestrial N budgets. For the CO<sub>2</sub> treatment, we found no evidence supporting increased recovery of free AAs in fine roots under elevated CO<sub>2</sub>. As a result, loblolly pine trees need to acquire additional N by other mechanisms, such as increased N use efficiency or increased root exploration.

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